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RECEPTOR BINDING AND MEMBRANE TRANSPORT OF BOTULINUM TOXINS

ANNUAL REPORT

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The goal of this research is to characterize the interaction of the botulinum type neurotoxins with target membranes and seeks to add to the field of study of toxin/cell interactions. Also, this research project seeks to determine any similarity between the mechanism of action of botulinum toxins and a number of other protein toxins whose mechanism of action has been more clearly defined. Through the utilization of antibodies to a series of diverse protein toxins and an artificial liposome target system for the botulinum toxin, it is hoped that the mechanism of action of the toxin can be better defined. Also, this will allow us to gain an understanding of how to inhibit the action of this toxin.

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FOREWORD

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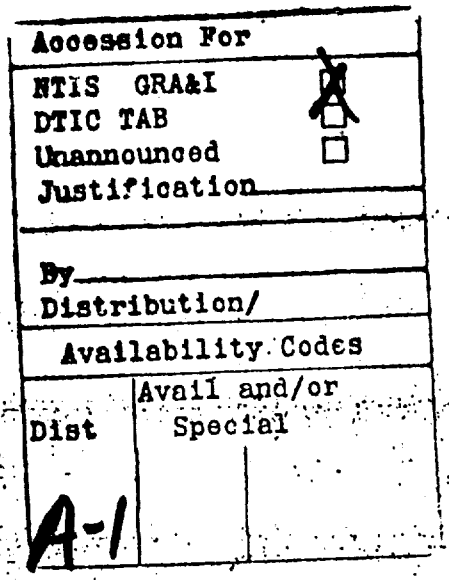

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Introduction

Antibodies to the "generalized" or model toxin molecules such as complement component C9, diphtheria, tetanus, colicin E1 and mellitin have been tested by us for cross-reactivity with the botulinum toxin molecule. A cross-reactivity between human C9 and melittin (1), both in terms of simple recognition and in the ability to inhibit membranolytic activity, has been demonstrated. As the source of these two proteins is quite diverse (human serum vs. bee venom) the ability to demonstrate a common antigenic site and a similar inhibition of function by a single antibody type points to the possibility that a common "lytic" mechanism exists for these two proteins. It is possible that the botulinum toxin and another, unrelated protein toxin could show a similar common "inhibitable" activity that could help to resolve the mechanism of action of botulinum. The antibodies will be tested for the ability to cross-reactivity with botulinum toxin to determine similarities in sequence (as yet undefined for botulinum toxin) and function.

A multi-domained model for the botulinum toxins has been proposed (2). In general, the proteins are composed of a polypeptide containing a heavy (MW≈100,000) and a light (MW≈50,000) chain that requires proteolytic cleavage before becoming fully toxic (2). In order for any toxin molecule to exert an effect against a target cell it must accomplish a minimum of three objectives: 1) bind to the target cell; 2) translocate inside the target; and 3) express a lethal activity. As the protein is also water-soluble it is realized that the molecule must also (in the translocating step) reorganize in order for it to cross the lipid barrier of the target cell. Other proteins that must accomplish these same three objectives, it could be argued, could perform the tasks in similar ways.

Through these studies it may be possible to better understand the mechanism of action of the botulinum toxins. Any common cross reactivities found between the botulinum toxin protein and another toxin molecule could point to similarities in any one of the following three generalized functions of these toxins: i) the enzymatic unit, ii) the membrane inserting or translocating domain, and iii) the receptor binding domain. As the protein sequences between these diverse proteins is not known to contain any direct analogies (and all the protein sequences have not been defined) and no direct antigenic cross-reactivities has been reported, we began to search for common cross-reactivities during the time the toxin molecules bound to or crossed artificial target cells (liposomes).

Materials and Methods

Supplies. Sources for all chemicals and reagents for buffers and solutions unless otherwise stated were purchased from Fisher Scientific. Dyes for the fluorescence studies from Molecular Probes, Inc. Lipids were obtained from Avanti Polar Lipids, and all electrophoretic chemicals were purchased from Serva. Botulinum toxin (Type B) was obtained from Calbiochem.

Lipid Vesicle Formation. Large unilamellar vesicles (LUV) and small unilamellar vesicles (SUV) were formed as described in (3,4).

Acid Shock Procedure. Vesicles in 10 mM Imidazole buffer containing 150 nM NaCl, pH 7 (10 μg lipid/mL) were subjected to a drop in pH by the addition of 10 μL of a 10% stock solution of succinic acid at pH 3.9. After 1 min the entire mixture was diluted into 3 mL of buffer at pH 7.4.

Antibody Preparation. Polyclonal IgG from selected animal sera will be isolated as described (5) and the final IgG fractions will be dialyzed against 10mM Tris-HCl, 150mM NaCl, pH 7.3.

Fluorescence Measurements. Determination of release of the dye carboxyfluorescein (CF) from lipid vesicles was performed by forming the vesicles in the presence of a self-quenching concentration of the dye molecule (100 mM). The free dye molecules were separated from the internalized dye and liposomes by either passing the mixture over a Sephadex G-100 column (collecting the void volume fractions of the column for the vesicles) or by repeated centrifugations (at least 3 times) with resuspension into dye-free buffer. The vesicles were then used within 4 hours for all assays of toxin activity. Measurement of dye release was performed on an Aminco Bowman Spectrophotofluorometer, adapted with a thermostatted, stirred cuvette, as described in reference (6). pH shock was performed as described above using 10% succinic acid, followed by the addition of 10 μL of a 1 M Imidazole, pH 8 stock solution used to return the final pH to a value of 7.

Immuno-blotting. The Laemmli system (7) was used to prepare the acrylamide gels for separation. Immunoblotting was performed using a Bio-Rad gel electrophoresis and transfer unit. Blots were developed using one of the various antibodies as a primary with alkaline phosphatase labeled secondary antibody used according to the manufacturer.

Results

A study of the antigenic similarities between the botulinum toxin molecule and various other protein toxin molecules was undertaken to determine if any antigenic similarities existed between botulinum and other specific toxins. Our goal was to determine if an antibody to a toxin that has had it's mechanism of action defined could recognize a portion of the botulinum toxin. It would be unlikely that a random cross-reactivity would exist between protein molecules as diverse as bacterial botulinum, diphtheria, and colicins and such toxins as melittin from bee venom and complement component C9 from human serum unless it was to some common structural or functional domain.

Investigation into the simlilarity of antigenic determinants of the botulinum toxin with polyclonal antibodies to the toxin molecules colicin E1, melittin (bee venom), and complement component C9 (human serum) have proven to be negative, in that no binding of any of the mentioned polyclonal antibodies was found that could recognize the botulinum toxin molecule after SDS-PAGE gels and transfer to nitrocellulose paper (Immunoblots. not shown).

Since polyclonal antibodies to the proteins: Human C9, Human C8, and melittin (from bee venom) generated in rabbits could not recognize botulinum toxin as determined by immunoblotting (Western Blots), the ability of these antibodies to inhibit toxin activity was examined. As stated previously, if the botulinum toxin underwent some transitional state during the activation of the toxin that ressembled a structural feature of one of the antigens above, it is possible that such an intermediate could be recognized by the antibodies with subsequent inhibition of activity. The action of botulinum toxin on artificial membrane targets was re-investigated using the release of an entrapped dye molecule (carboxyfluorescein) as an indicator of membrane damage. This procedure has been described previously by us (see earlier reports). The composition of the liposome targets was 70% DPPC and 30% DPPS which we have found to be most effective in allowing the botulinum toxin to express a pH-dependent membranolytic activity (Annual Report, 1988, Table 1). It was reasoned that the ability of the antibodies to recognize the botulinum toxin transferred to nitrocellulose paper (as for immunoblots) might be distinct from an ability to recognize a site(s) on the toxin molecule in solution or upon interaction with a membrane surface. An ability of an antibody to inhibit the membranolytic activity of botulinum toxin in this assay could indicate a possible common antigenic site. Polyclonal antibody to human C9 was tested for an ability to inhibit the activity of botulinum toxin as described above, using antibody concentrations such that as much as a 100 to 1 (mole:mole) ratio of antibodies to botulinum toxin was acheived. Shown in Table 1 below is the effect of antibodies to C9 on the pH-dependent release of dye molecules from liposome targets in the presence of botulinum.

Table 1. Effect of Antibodies to human C9 to inhibit the membranolytic activity of botulinum toxin*.

Antibody Added, amount	% Dye Release
None	93 ± 12
anti-C9, 1 ug	97 ± 13
anti-C9, 10 ug	80 ± 18
anti-C9, 50 ug	75 ± 15

**Conditions: 10 g of liposomes and 0.5 ug botulinum toxin were mixed 30 secs prior to the acid shock and determination of dye release. When present, antibodies were added prior to toxin addition.*

The small reduction in the activity of botulinum toxin with the large excess (50 ug) of antibody to C9 does not indicate that a significant inhibiton was taking place under these conditions.

The ability of antibodies to several membrane active proteins from diverse sources were used to test their ability to inhibit the membranolytic activity of the botulinum toxin as described previously. Thus far, polyclonal antibodies to these proteins have been unable to affect the pH-dependent activity of botulinum toxin on lipid vesicles. At high antibody concentrations (>50 ug per 0.5 ug botulinum toxin), however, some small reduction in the activity of the toxin can be detected using anti-C9 and anti-melittin polyclonal antibodies. Polyclonal antibodies to melittin were more effective than antibodies to C9 in that 50 ug of antibody to melittin was somewhat more effective in reducing the membranolytic activity of botulinum toxin.

Table 2. Inhibition of the pH-dependent Membranolytic Activity of Botulinum Toxin by Polyclonal Antibodies in DPPC/DPPS Liposomes.

100 ug antibody to:	% Dye Release from Liposomes
None	93 ± 14
Human C9	77 ± 15
Human C8	91 ± 15
Hen Lysozyme	97 ± 11
Bovine Albumin	93 ± 13
Melittin (Bee)	72 ± 16

The influence of liposome composition on the inhibitory activity of the antibodies against the toxin was also examined. Although the relative activity of the toxin against the various liposome targets is not the same (see Annual Report, 1988) it could be of value to determine if any toxin activity could be inhibited under these conditions (i.e., Botulinum toxin-mediated pH-dependent dye release).

Table 3. Effect of Liposome Composition on the Ability of Anti-Human C9 to Inhibit the Membranolytic Activity of Botulinum Toxin. Antibody/Toxin = 100 ug/0.5 ug.

Liposome Composition	% Inhibition of Dye Release
Asolectin	6
DMPC	4
DMPC/DOPC (50/50)	12
DPPC/DOPC (50/50)	0
DMPC/DPPS (70/30)	7
DPPC/DPPS (70/30)	18
Egg PC	0

In order to augment any botulinum-inhibiting activity of the polyclonal antibodies we will construct an affinity column to purify the polyclonal antibodies. Botulinum toxin will be coupled to sepharose beads and used to separate those antibodies (in the polyclonal population) that bind to the botulinum toxin. We have used this procedure in the past to purify other reagents, such as antibodies to human C9. It is hoped that passage of the polyclonal antibodies over this "fixed" botulinum toxin will allow antibodies that do not recognize the toxin to pass through leaving only bound antibodies. Antibody that does recognize the toxin will be eluted by: high salt, 2 M $MgCl_2$, 2 M KBr, low pH, high pH. These conditions have been used by ourselves and others to break antigen-antibody complexes. If antibody is not recovered, harsher techniques will be used (8 M Guanidine-HCl), although this will be a last resort as it is hoped that the elution conditions will not destroy the ability of the antibody to recognize an antigen.

Discussion

Plans for the next phase of investigation will include the screening of antibodies to complement component C8, diphtheria, ricin, and cholera toxin for any cross reactivity to the botulinum toxin. Any similarity found in binding of these antibodies from such diverse sources would argue that any cross-reactivity could be due to similarities in the functional domains of the molecules.

The inability of the antibodies to other toxin-like of toxic molecules thus far to inhibit the activity of botulinum toxin could be due to the conditions required to activate the botulinum toxin (i.e., low pH). To address this this we will use column-coupled sepharose (as described above) to purify our antibody populations in such a way as to select for those antibodies that only bind at pH 4. These antibodies, once eluted from the column (by either raising the pH or different salts as described above) will be used in the in vitro assay of toxin activity.

The antibodies will also be screened for their ability to inhibit botulinum activity from inside the lumen of the liposome. Our ability to perform these experiments are now underway, but we are having difficulty entrapping a sufficient amount of antibody while at the same time retaining liposome integrity at the low pH values.

A novel method to produce inhibitory peptides is being proposed for small peptide hormones by other workers (8). In one case, arginine vasopressin (AVP) is being used as a model to demonstrate the feasibility of producing artificial inhibitory peptides to this hormone. This method involves the use of an algorithm which bases selection of the "inhibitor's" amino acid sequence loosely upon pairing of hydrophobic and hydrophilic residues between AVP and the inhibitor. The proposed peptide is then manufactured synthetically by this same laboratory (9). This technique is currently being performed at the University of Florida.

These approaches to study both the similarity between botulinum toxin and other toxins and the production of artificial inhibitors to botulinum toxin will provide a powerful addition to our study of the protein in liposomal targets.

The possibility to produce an artificial inhibitor to botulinum toxin could be most important. The reported ability of artificially generated peptides to inhibit small peptide hormones has been demonstrated. As this provides evidence that hormonal signals can be inhibited which normally are provided at very small concentrations ($< 10^{-12}$ M) it is hoped that a toxin as potent as botulinum toxin may also be inhibited as effectively. As it is now, an antibody response must be developed against the toxoid molecule which may be incompletely effective in providing a defence against the toxin and which takes weeks or months to develop. An artificial toxin inhibitor would provide an additional force to be used against the toxin, either alone or while an antibody is being developed, and would also provide a useful tool in the study of the mechanism of action of the protein in artificial systems of study.

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